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Urokinase: Isolation, Purification, Characterization, New Spectrophotometric Bioassay Method and In-vitro Blood Clot Dissolving Activity from Cow Urine

Research article

Badhe Ravindra V[1*], Badhe Sonali R[2], Bhujbal Mayur N[1], Nanda Rabindra K[1], Shirolkar Satish V[1.]

1. Pharm. Chem. Dept., Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune – 411018.
2. Pharmacology Dept. Modern College of Pharmacy, Yamunanagar, Nigdi, Pune 411044.

Abstract

Urokinase, a potent plasminogen activator is an enzyme present in cowurine attributing to the thromboembolic properties of cow urine but its presence in cow urine has never been convincingly documented. In our work, we did isolation, purification, partial characterization and quantitation of urokinase in cowurine using newly developed spectrophotometric bioassay method and studied its invitro blood clot dissolving activity. The cow urine was collected from cows of Indian origin (*Bos indicus*). Isolation of Urokinase was done by ammonium sulfate precipitation and dialysis. Purification was done by using Sephadex G-200 Gel Filtration Chromatography. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Gelatin Zymography, HPLC and TLC of tryptic digest of the purified urokinase was carried out for partial characterization studies which conformed the molecular weight of 59 kD of the purified Urokinase. Quantification with the help of new spectrophotometric bioassay method showed 956 IU/ml and with the help of HPLC it was 910 IU/ml of urokinase in cow urine. The invitro blood clot dissolving activity on a cotton cloth confirmed the activity and efficacy of the isolated and purified urokinase.

Keywords: Cow urine, Plasminogen activator, New Spectrophotometric Thrombolytic Bio Assay, Urokinase, In-vitro clot dissolving activity, Tryptic digest fingerprint.

1. Introduction:

Cow urine from Indian cows (*Bos indicus*) has a unique place in Ayurveda and has been described in many ancient manuscripts like Atharva Veda, Charaka Samhita, Rajni Ghuntu, Vridhabhagabhatt, Amritasagar, Bhavaprakash, Sachitra Ayurveda, and Sushruta Samhita to be the most effective substance / secretion of animal origin with therapeutic values. It has been recognized as 'Water of Life' or 'Amrita'. In India, drinking of cow urine has been practiced for thousands of years (1-3).

Cow Urine Treatment And Research Center, Indore, and Go Vigyan Anusandhan Kendra, Nagpur, India has conducted a lot of research over the past few years and has reached the conclusion that it is capable of curing thromboembolic diseases, diabetes(4), blood pressure, asthma, psoriasis, eczema, heart attack, blockage in arteries, fits, cancer (5), Immunomodulatory (6) piles, prostrate, arthritis, migraine, thyroid, ulcer, acidity,

*Corresponding Author:
Badhe Ravindra V
Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune-18.
Tel: +91-9422432038,
Fax no.: 020-27420261
Email: badheravi@rediffmail.com

constipation, gynecological problems, ear and nose problems and several other diseases (3, 7-12).

The analysis of cow urine has shown that the major chemical composition is Urea, Uric Acid, Enzyme-Urokinase, Insulin, Nitrogen, Sulphur, Phosphate, Sodium, Manganese, Carbolic acid, Iron, Silicon, Chlorine, Magnesium, Melci, Citric, Titric, Succinic, Calcium salts, Vitamin A, B, C, D, E, Minerals, Lactose, Creatinine, Hormones and Swarna Kshar (13)

Urokinase (UK; EC 3.4.21.73), a plasminogen activating proteolytic enzyme, was first described in 1951 as occurring in trace quantities in mammalian urine. The Urokinase detected in urine is produced by kidney cells. Urokinase catalyzes the conversion of plasminogen to plasmin by cleaving the Arg-Val linkage in the Pro-Gly-Arg- Val sequence of the former. As the resulting plasmin dissolves clots of fibrin in blood vessels, Urokinase is intravenously administered for treatment of thromboembolic disease (14).

Thromboembolic diseases are today a major cause of morbidity and mortality. Thrombolytic enzymes have apparent significance in thrombosis therapy and great attention has been directed towards a search for thrombolytic agents of various origins with particular reference to agents with more specificity and less toxicity. The thromboembolic diseases, heart attacks, blockage in arteries cured by consumption of cow urine and cow urine products are due to the presence of the enzyme Urokinase in cow urine. Therefore more research is needed to be directed on the isolation of Urokinase from cow urine considering its medicinal and therapeutic value. Our studies are focused on isolation, purification, partial characterization and quantitation of Urokinase present in Cow urine.

The present work describes the isolation, concentration, purification of Urokinase from freshly collected samples of Cow urine from cows of Indian origin, its partial characterization and quantitation by using novel spectrophotometric thrombolytic bio assay method and studying the in-vitro blood clot dissolving activity of the cow urine.

2. Material and Methods:

Sample collection: Cow Urine was collected from cows of Indian origin. Dialysis membrane: HIMEDIA. (Cut off 10-12 kD). UV–Visible Spectrophotometer: Shimadzu UV-1700. Medium range protein molecular weight marker (94.7 kD-14.3kD): Bangalore Genei Private Ltd. (Banglore, India). Haemostasis reagent: rabbit brain thromboplastin reagent (Diagnos Thrombo 1.0). All other chemicals were of analytical reagent grade

2.1. Collection of Cow urine.

Cow Urine was collected from cows of Indian origin (*Bos indicus*). Physico-chemical properties of Cow Urine: Colour: dark yellow, Odour: very strong (Ammonia), pH: alkaline (8-9.5)

2.2. Purification of Urokinase from Cow urine:

2.2.1. Ammonium Sulphate Precipitation

The concentration of ammonium sulfate was optimized to 70-80% of saturation to give maximum protein precipitation. All steps were carried out at 2-8 °C. The precipitated proteins were centrifuged (15).

2.2.2. Dialysis:

The protein pellets formed after centrifugation were resuspended in 10 ml of 0.1M Tris-HCl buffer, pH 7 and tied in the dialysis membrane in small pouches and were kept immersed in beaker containing 0.1M Tris-HCl buffer, pH 7 (1000 ml) and dialysis was carried out. The dialysis beakers were kept on magnetic stirrer so that continuous concentration gradient was maintained.

The temperature was maintained at 2-8 °C for the whole process (15).

2.2.3. Sephadex G-200 Gel Filtration Chromatography

The dialyzed protein mixture was loaded onto a column of Sephadex G-200 (1.5 × 30 cm) (Sigma-Aldrich, St Louis, MO) equilibrated with 0.1M Tris-HCl buffer, pH 7. The column was eluted at a flow rate of 60 mL/hrs with a gradient of 0.1M to 1M NaCl in the same buffer. Urokinase eluted out was quantified by using novel spectrophotometric thrombolytic bio assay method. Fractions with high thrombolytic activity were pooled, dialyzed, concentrated and used for further studies. (16, 17)

2.2.4. Protein Assay

Protein was measured by the method of Lowry et al with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was calculated from the absorbance at 680 nm. (18)

2.2.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis:

After Sephadex G-200 column chromatography, the pooled concentrated fractions showing the highest specific activity were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed according to Laemmli using 12.5 % gel. (19)

2.2.6. Gelatin Zymography:

After Sephadex G-200 column chromatography, the pooled concentrated fractions showing the highest specific activity were subjected to gelatin Zymography which is an electrophoretic technique, based on SDS-PAGE, that includes gelatin copolymerized with the polyacrylamide gel, for the detection of enzyme activity. (20)

2.2.7. RP-HPLC method:

Optimized mobile phase consisting of 0.06% TCA in water: Acetonitrile (45:55% v/v) was selected as it gave good spectra with minimum retention time and minimal tailing. It is ultrasonicated for 20 minutes, filtered through a 0.45 µ membrane filter and degassed under vacuum. The urokinase stock solution was prepared of 2,000 IU in 20 ml of mobile phase to obtain stock solutions of concentration of 200 IU/ml of urokinase.

Column used was Kromasil C_{18} column (25cm (L) X 4.6 mm ID), Column temperature was ambient, Flow rate was maintained at 1.0 mL min^{-1}, Run time was 5 min, UV detection at 215 nm and Injection volume was 20 µl.

Standard Calibration Curves of urokinase was prepared for standard urokinase dilutions ranging from 400 to 2000 IU/ml and the isolated purified urokinase was injected with suitable dilution.

2.2.8. Tryptic digest - TLC fingerprint:

The standard and purified urokinase was enzymatically degested with the help of Trypsin as per the method of Flannery (21). Comparative TLC fingerprint was developed on silica plates with concentration 5 mg/ml and the optimized mobile phase used was 1-butanol / pyridine / ammonia (25%) / water (37 / 32 / 8 / 23).

2.3. Thrombolytic activity:

2.3.1. Novel Spectrophotometric Thrombolytic Bio Assay Method:

1% w/v solution of calcium chloride is mixed with plasma it results in formation of clots within 5min. The rate of suspended clot formation is increased by addition of rabbit brain thromboplastin. The absorbance of this suspended clot was adjusted at 0.2 ± 0.01 absorbance at λ_{max} 530 nm by addition of sufficient amount of distilled water. The linear decrease in the absorbance of the clots was taken by

addition of Urokinase standard solution from 200-1200 IU/ml to 5 ml of clot solution. The level of urokinase in cow urine and that purified from cow urine was calculated in IU/ml from the calibration curve by addition of specified quantity of purified urokinase to 5ml of clot solution. (22)

2.3.2. In-vitro blood clot dissolving activity of Cow urine and Purified urokinase.

The in-vitro blood clot dissolving activity of cow urine and purified urokinase was studied on 3 white cotton cloth pieces (10 × 10 cm) stained with blood. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied:

1. Flask with distilled water (100 mL) + stained cloth (cloth stained with blood)
2. Flask with Cow urine (100 mL) + stained cloth (cloth stained with blood)
3. Flask with distilled water (100 mL) + stained cloth (cloth stained with blood) + 2 mL urokinase solution (5000 IU/ml)

The above flasks were incubated at 37°C for 120 minutes. After incubation, cloth pieces were taken out, rinsed with water, and dried. Visual examination of various pieces proved the effect of enzyme in removal of blood clots. Untreated cloth piece stained with blood were taken as control. (16)

3. Result:

3.1 Concentration of urokinase activity in starting material.

In present study urokinase was isolated, concentrated, purified, partially characterized and quantified from freshly collected samples of Cow urine of cows of Indian origin. We have made an attempt to find out the concentration of urokinase in cow urine prior to its further purification. The yield of urokinase was found out to be 110 IU/ml in cow urine prior to further purification of urokinase. This was calculated using the newly developed Novel Spectrophotometric Thrombolytic Bio Assay Method calibration curve for estimation of urokinase. (Fig 1)

3.2 Ammonium sulfate precipitation and Dialysis.

The concentration of ammonium sulfate was optimized between 70-80% to get the maximum amount of protein precipitation from the cow urine samples. The concentration of urokinase in the precipitated and dialyzed fraction was calculated out by using the newly developed Novel Spectrophotometric Thrombolytic Bio Assay Method calibration curve for estimation of urokinase. (Fig 1) The concentration of urokinase was found out to be 316 IU/ml.

3.3 Sephadex G-200 Gel Filtration Chromatography.

The protein pellet obtained after 70-80% saturation with ammonium sulphate and dialysis was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-200 equilibrated with 0.1M Tris-HCl buffer, pH 7. The elution profile of gel filtration chromatography is shown in Fig 2.

The eluted urokinase was quantified by using thrombolytic bio assay method and fractions (15-20) with greater thrombolytic activitie at 0.6 M NaCl conc. were pooled, dialyzed, and concentrated by lyophilization and used for further studies. The summary of purification steps involved for urokinase from cow urine is presented in Table 1.

Table 1. Summary of Purification Steps of Urokinase

Fraction	Total Enzyme Activity IU	Total Protein (mg)	Urokinase Activity IU/ml	Fold Purification	% Recovery
Cow Urine	9350	85	110	1	100

$(NH_4)_2SO_4$ precipitation, dialyzed	8532	27	316	2.87	91
Sephadex G-200	5162	5.4	956	8.69	52

3.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis:

The purified pooled urokinase fraction was analyzed by SDS-PAGE, the result observed consisted of a single polypeptide chain of high molecular weight urokinase with an apparent molecular weight of 59 kD band in Lane A. (Fig 3). The molecular mass standards were Phosphorylase b 97.4 kD BSA (66 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), trypsinogen (20.1 kD), and Lysozyme (14.3 kD) on SDS-PAGE Lane B. The molecular weight was determined by interpolation from a linear semilogarithmic plot of relative molecular mass versus the R_f value (relative mobility).

SDS-PAGE of pooled peak fractions eluted from the Sephadex G 200 column. **Lane A** contains the 59 kD band of single chain high molecular weight urokinase chain taken from the pooled fraction. **Lane B** contains the medium molecular marker used as the standard on a 12.5% SDS-PAGE.

3.5. Gelatin Zymography:

To test the proteolytic property of the urokinase eluted in the peak pools, Zymographic analysis was performed (Fig. 4) using reference urokinase as the positive control. The pooled fraction produced detectable zones of lysis on the Zymogram (Fig. 4, Lane A) in the molecular range of 59 kD which corresponded to the band observed in the SDS-PAGE pattern. This indicated high concentration of urokinase.

Zymogram of pooled peak fractions eluted from the Sephadex G 200 column. Protein (20 µg) was loaded in Lane A and B and electrophoresis carried out. Lanes: (A) pooled purified urokinase; (B) urokinase (U-FRAG). Clear zones of lysis indicate bands of urokinase.

3.6. In-vitro blood clot dissolving activity of Cow urine and Purified urokinase.

The blood clot was taken on a white cloth shown in Fig 5A, the cloth was then washed with distill water which was taken as control Fig 5B. Then the cloth was washed with cow urine and purified urokinase from Sephadex G 200 Fig 5C and D respectively. The visual results for the blood clot dissolving activity of both cow urine and purified urokinase showed that they possessed fibrinolytic property. The clot dissolution effect of purified enzyme was considerably high as compared to cow urine as whole. (Fig 5)

3.7 RP-HPLC method:

The chromatogram of standard as well as purified urokinase was developed on the RP-HPLC column with optimized mobile phase. The retention time was found out to be 1.87 mins.(Fig 6a) The Beer-Lambert's law was obeyed in the concentration range of 400-2000 IU/ml for urokinase. The prepared calibration curve have equation y=19810x and r^2=0.9923 which was used to calculate the amount of isolated urokinase in cow urine and was found to be about 910 IU/ml (Fig 6b).

3.8 Tryptic digest - TLC fingerprint:

The fingerprint chromatogram of standard as well as purified urokinase tryptic digest was developed on the silica gel TLC with optimized mobile phase. The Rf values found to be 0.130, 0.270, 0.423, 0.530, 0.707, 0.900 respectively for both standard and purified urokinase which indicated the isolated protein was urokinase (Fig 7).

4. Discussion:

Despite the immense clinical application of Urokinase present in Cow urine in the treatment of thromboembolic diseases, no satisfactory research has yet been described for purification of Urokinase from cow urine. In this study, conventional methods were initially tried for concentration and partial purification of the urokinase from cow urine, like ammonium sulphate precipitation, dialysis, Sephadex G-200 Gel Filtration Chromatography. SDS-PAGE analysis of the eluates from ion exchange column revealed single polypeptide high molecular weight band at 59 kD of urokinase as confirmed by Zymography. This was in agreement with the presence of these bands between 58.5±5 kD with reference to the work of previous researchers wherein different high molecular weight forms of urokinase have been reported.

Prior to the ion-exchange chromatography step, attempts were made to concentrate urokinase activity in the cow urine. For this purpose, classical techniques of ammonium sulfate precipitation, dialyses were tried and optimized. The principle of the Novel Spectrophotometric Thrombolytic Bio Assay Method was utilized to quantify the level of urokinase present in cow urine at various levels of purification which was found to be relatively higher 956 IU/ml as compared to ammonium sulfate precipitated and dialyzed sample as well as cow urine itself which were 316 IU/ml and 110 IU/ml respectively (Table 1). HPLC method confirmed the presence of urokinase to 910 IU/ml which is very close to the value by bioassay. Tryptic digest TLC fingerprint confirmed the isolated and purified protein was urokinase

4. Conclusion:

In conclusion, in this paper we have made an attempt to investigate the presence of urokinase in cow urine and carry out its partial characterization and quantification by our newly developed Novel Spectrophotometric Thrombolytic Bio Assay Method. The in-vitro clot dissolving studies confirmed the claim of Ayruveda of thrombolytic activity of cow urine and other cow urine products. Further investigations are needed to be done by studying the different marketed formulations of cow urine in relation to the activity of urokinase.

5. Acknowledgement

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Fig1: Calibration Curve of Bioassay of Urokinase. Absorbance vs. concentration (IU)

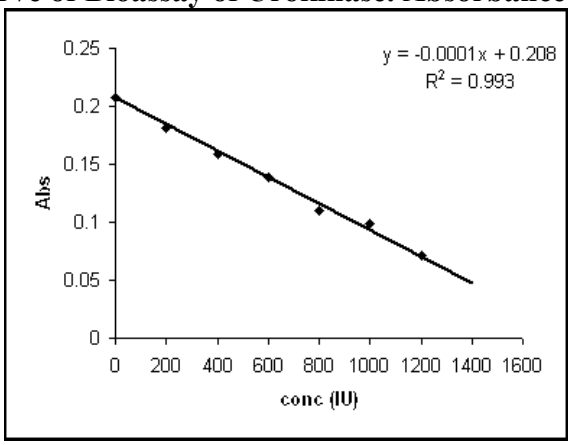

Fig2. Elution profile of Urokinase by Sephadex G-200 column.

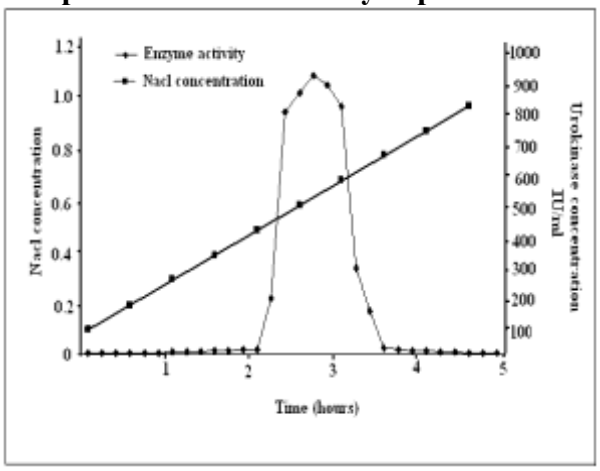

Fig3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

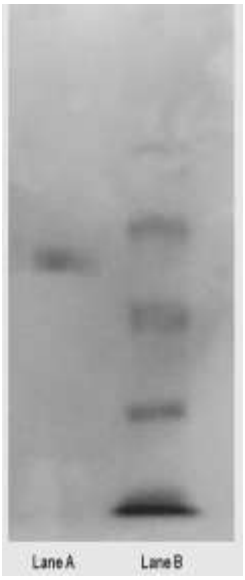

Fig 4. Gelatin Zymography

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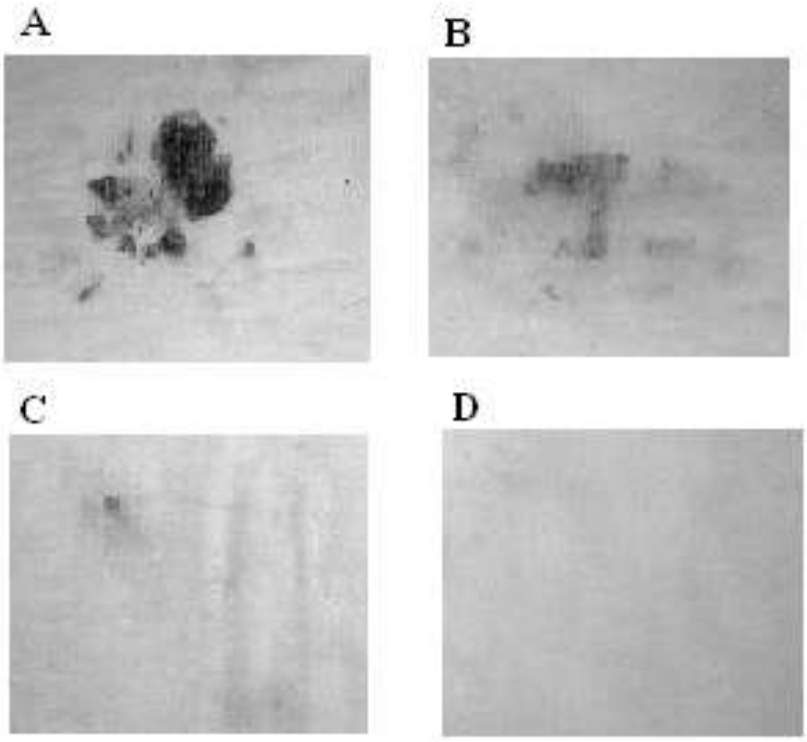
Fig 5. In-vitro blood clot dissolving activity of Cow urine and Purified urokinase.



A. Cloth Stained with Human Blood
B. Blood Stained Cloth Washed With Water
C. Blood Stained Cloth Washed Cow urine
D. Blood Stained Cloth Washed With Water + Purified Urokinase (5000 IU/ml)

Figure 6a. Chromatogram of standard urokinase

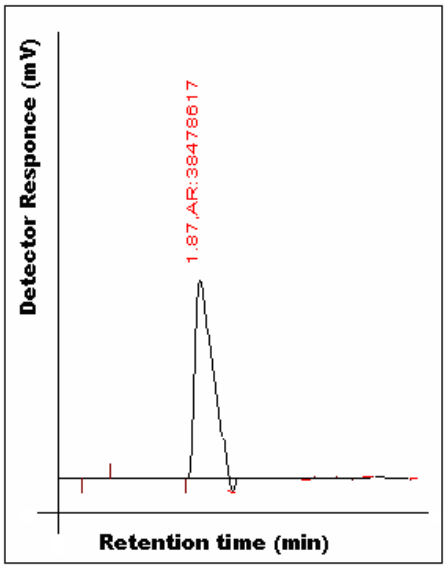


Figure 6b. Standard Calibration curve for urokinase by RP – HPLC

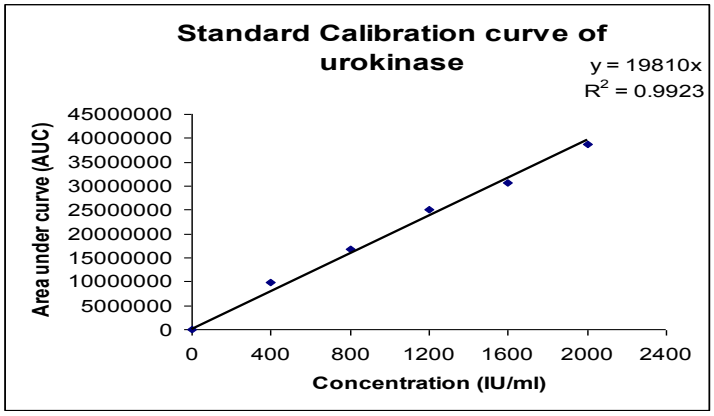


Fig 7. Rf values and Tryptic Digest Fingerprint of Purified (P) and Standard (S) Urokinase.